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Characterization of an experimental model of neonatal meningitis induced by Group B Streptococcus

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Mestrado em Bioquímica
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O Presidente do Júri,

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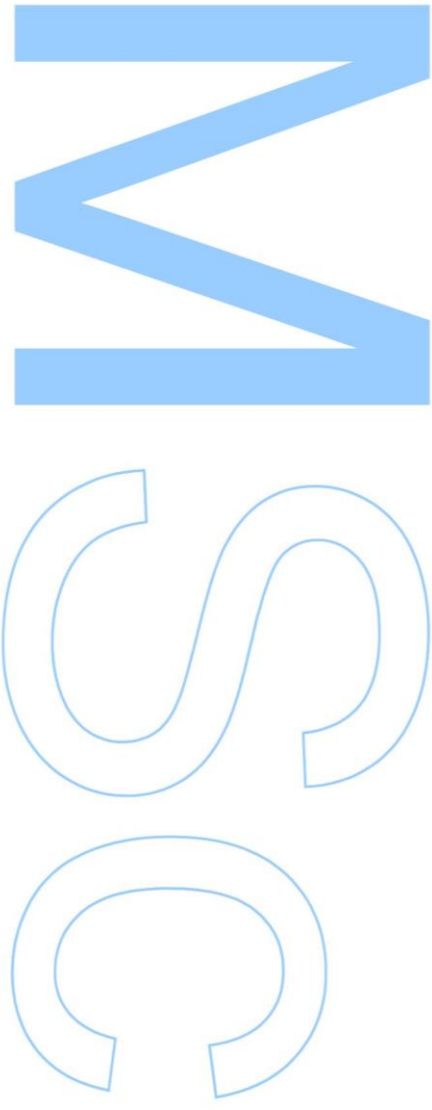

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“If you're going to try, go all the way. Otherwise, don't even start. This could mean losing girlfriends, wives, relatives and maybe even your mind. It could mean not eating for three or four days. It could mean freezing on a park bench. It could mean jail. It could mean derision. It could mean mockery - isolation. Isolation is the gift. All the others are a test of your endurance, of how much you really want to do it. And, you'll do it, despite rejection and the worst odds. And it will be better than anything else you can imagine. If you're going to try, go all the way. There is no other feeling like that. You will be alone with the gods, and the nights will flame with fire. You will ride life straight to perfect laughter. It's the only good fight there is.”

— Charles Bukowski, *Factotum*

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Espero que estejam tão orgulhosos e felizes quanto eu, porque esta tese é dedicada a vocês, família. Muito obrigada!

Resumo

A meningite neonatal induzida por bactérias é uma importante causa de mortalidade e morbilidade em todo o mundo. *Streptococcus agalactiae,* mais comummente designado por *Estreptococos* do Grupo B (do Inglês GBS) é o responsável pela maioria dos casos de meningite neonatal. A colonização materna dos tratos gastrointestinal e genitourinário são a principal fonte de infeção dos recém-nascidos. Apesar das melhorias nos cuidados neonatais e da implementação de medidas antimicrobianas, a taxa de mortalidade associada a este microrganismo é de 10%. Além disso, até cerca de 50% dos sobreviventes da meningite causada por GBS desenvolvem graves sequelas neurológicas. Ainda existe uma grande lacuna no conhecimento da patogénese desta doença, apesar da existência de vários modelos de experimentação animal de meningite neonatal. Uma possível explicação é que nenhum destes modelos utiliza a via de infeção que ocorre nos humanos. No nosso laboratório estamos a desenvolver um modelo que mimetiza a via de infeção humana. Neste sentido, fêmeas BALB/c grávidas foram infetadas intra-vaginalmente com 10^5 células de GBS BM110, estirpe responsável pela maioria dos casos de meningite humana. Após o parto, a colonização do trato vaginal das fêmeas e a carga bacteriana presente nos descendentes foram monitorizadas em diferentes tempos. Os resultados obtidos mostraram que as fêmeas estavam altamente colonizadas até ao quarto dia após o parto e que, a bactéria foi transmitida às ninhadas. De fato, GBS foi encontrado nos pulmões, no sangue, no fígado e no cérebro dos ratinhos recém-nascidos, a diferentes tempos após o parto. Análise histopatológica dos cérebros de ratinhos infetados mostrou que estes apresentavam as caraterísticas típicas de meningite: espessamento das meninges, hemorragias cerebrais e influxo de células inflamatórias. Aproximadamente 40% da descendência morreu após o parto, um valor semelhante ao descrito em humanos antes da implementação da profilaxia antibiótica *intrapartum*. De modo a confirmar se os animais sobreviventes à semelhança do descrito em humanos apresentavam sequelas neurológicas, foram avaliados, na idade adulta, o seu desempenho cognitivo e motor bem como os padrões de neurotransmissores em diferentes secções de cérebro. A avaliação cognitiva foi determinada usando o *Radial Maze Test*, e a atividade locomotora e comportamento exploratório foram avaliados usando o *Open Field Test*. Após os testes, os animais foram sacrificados e diferentes secções dos seus cérebros foram utilizadas para medir as concentrações de neurotransmissores. Ratinhos sobreviventes à infeção por GBS apresentaram um maior número de erros cometidos quando comparados com os controlos. Estes erros

estão associados com os níveis de glutamato no hipocampo que se encontravam diminuídos no grupo de ratinhos que sobreviveram à infeção. Os resultados obtidos no *Open Field Test* indicaram que estes ratinhos são menos ativos, uma vez que apresentaram atividade locomotora e comportamento exploratório diminuídos quando comparados com o grupo controlo. Estes resultados estavam associados com os níveis diminuídos de glutamato no tálamo e com os níveis diminuídos de dopamina e seus metabolitos no hipocampo e no estriado dos ratinhos sobreviventes, quando comparados com os controlos. Foram assim observadas alterações neurológicas nos ratinhos que sobreviveram à infeção neonatal por GBS.

Em conjunto, estes resultados indicam que o nosso modelo mimetiza o que acontece nos humanos, sendo por isso um bom modelo para ser usado na caracterização da patogénese e patofisiologia da meningite induzida por GBS.

Palavras-Chave: Meningite neonatal; *Streptococcus* do Grupo B; modelo experimental; neurotransmissores; comportamento

Abstract

Bacterial meningitis is a substantial cause of morbidity and mortality in neonates. Group B *Streptococcus* (GBS), a common designation for *Streptococcus agalactiae,* is the main agent of neonatal meningitis. Maternal colonization with GBS in the genitourinary and/or gastrointestinal tracts is the source of the neonatal infection. Despite early antimicrobial treatment and improvement in neonatal intensive care, up to 10% of neonatal invasive GBS infections are lethal and up to 50% of surviving infants with meningitis present neurological sequelae. Significant gaps in knowledge of the pathogenesis of this disease still remain despite several animal models have been used. All these models use artificial routes of infection. Our research team is developing a murine model of neonatal GBS-induced diseases, which addresses the natural course in human pathogenesis of ascending infection from the lower genital tract to neonates. Here, we characterize the suitability of this animal model to study the neonatal meningitis induced by GBS. For that purpose, pregnant BALB/c female mice were infected intra-vaginally with 10^5cells of GBS BM110, a strain responsible for the majority of human cases of meningitis. Pregnant female were allowed to deliver and their vaginal tract colonization and the bacterial transfer into the newborns, were monitored at different time points after birth. The obtained results showed that mothers remained highly colonized until the fourth day after delivery and the bacterium was transmitted to their progeny. Indeed, GBS was found in the lungs, blood, liver and brain, of pups at different time points after birth. Histopathological analysis of the brains of infected pups showed the classical features of meningitis such as: meningeal thickening, cerebral bleeding and massive influx of inflammatory cells. Approximately 40% of the progeny died, a value close to that reported for the initial case-fatality observed in humans, before the introduction of *intrapartum* antibiotic therapy. To confirm that the surviving animals present neurological sequelae, we determined their cognitive and motor performance in adult life, and identified whether an altered neurotransmission pattern was involved. The cognitive evaluation was performed in a complex learning task using the Radial Maze test and the anxiety levels, locomotor activity and exploratory behavior were measured through Open Field Test. After the tests, the animals were sacrificed and the different brain regions were used for determination of neurotransmitter levels. Mice that survived to neonatal GBS infection presented increased working and reference memories errors when compared with the non-infected controls, which were associated with the decrease in the glutamate levels in the hippocampus. Results from Open Field Test, showed that mice survivors to GBS infection, were less active since they present decreased locomotor activity and

exploratory behavior than the non-infected controls. These results are associated with the lower levels of glutamate in the thalamus and also with the diminished levels of dopamine and its metabolites in the hippocampus and in the striatum of GBS-survivors comparatively with non-infected controls.

Altogether these results indicate that our mouse model closely mimics the characteristics observed in humans and, therefore, could be used to characterize the pathogenesis and pathophysiology of meningitis induced by GBS.

Keywords: Neonatal meningitis; Group B *Streptococcus*; experimental model; neurotransmitters; behavior

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Tables and Figures

Abbreviations

5-HIAA	-	5-hydroxyindolacetic acid
5-HT	-	Serotonin
ABC	-	Active Bacterial Core
BBB	-	Blood brain barrier
BCSFB	-	Blood Cerebrospinal Fluid Barrier
BECS	-	Brain Extracelular Fluid
BMEC	-	Brain MicrovascularEndothelial Cells
BSA	-	Bovine Serum Albumin
CAMP	-	Christie Atkins Munch-Petersen
CDC	-	Center for Disease Control
CFU	-	Colony-forming units
CNS	-	Central Nervous System
CP	-	Choroid Plexus
CSF	-	Cerebrospinal Fluid
E	-	Epinephrine
EOD	-	Early onset disease
DA	-	Dopamine
DOPAC	-	3,4-diydroxiphenylacetic acid
FbsA	-	Fibrinogen-binding protein A
G	-	Gestation
GABA	-	Gamma-Aminobutyric Acid
GAPDH	-	Glyceraldehyde 3-phosphate dehydrogenase
GBS	-	Group B Streptococcus
HBMEC	-	Human Brain Microvascular Endothelial Cells
HPLC	-	High Performance Liquid Chromatography
HPLC/ED	-	High Performance Liquid Chromatography with Electrochemical Detection
HVA	-	Homovanillic Acid
HvgA	-	Hypervirulent GBS adhesion
IAP	-	*Intrapartum* antibiotic prophylaxis
IgG	-	Immunoglobulin G
IL-10	-	Interleukin 10
LTP	-	Long Term Potentiation

LOD	-	Late onset disease
Lmb	-	Laminin-binding protein
MLST	-	Multi-locus sequence typing
MOI	-	Multiplicity of infection
NE	-	Norepinephrine
OF	-	Open Field
OFT	-	Open Field Test
PND	-	Postnatal Day
ST	-	Sequence type
ST-17	-	Sequence type 17
TH	-	Todd-Hewitt
WT	-	Wild type

Introduction

1. Neonatal Bacterial Meningitis

1.1 Overview

Neonatal bacterial meningitis is a serious life-threatening disease and a major cause of disability worldwide, despite advances in health care, in the development of more effective antibiotics, and in greater tools for rapid pathogen identification (Gaschignard et al., 2011).

At birth, all organ systems of the neonate switch from a highly controlled intra-uterine environment to the drastically different surroundings of the outside world.

This uttermost transition is then followed by a gradual, age-dependent maturation. Actually, prenatal immune system maturation cannot be fully conducted until neonates lose their status of privileged allograft, surrounded by a great number of maternal antigens, the fetus remains in his 'sterile world', unresponsive to any challenge. Yet, the immune system is prepared to cope with the dramatic changes that occur during birth, as newborns respond to microbes from the circling new non-sterile environment.

Nevertheless, neonates are at greater risk of developing infections, sepsis and meningitis when compared to other age groups as their immune system is not fully mature. Deficiencies in humoral and cellular immunity are setbacks that make them more susceptible to infections. The defects in adaptive immunity are well described [reviewed in (Adkins et al., 2004)], as they do not have immunological memory, are unable to produce high levels of IgG against encapsulated bacteria, produce less efficient inflammatory cytokines and also they are committed to produce the immunosuppressive/immunoregulatory cytokine IL-10 (Madureira et al., 2007, Madureira et al., 2011). Thus, neonates must rely on their innate immune system for protection against pathogens (Krishnan et al., 2003, Firth et al., 2005). Nevertheless, newborns have a diminished bone marrow pool, and therefore their ability to accelerate neutrophil production in response to infection is limited (Koenig and Yoder, 2004). Also, neutrophil recruitment in neonates is reduced (Koenig and Yoder, 2004). Moreover, the phagocyte function is diminished in neonates, especially in premature babies. (Falconer et al., 1995).

1.2 Epidemiology

The incidence value for neonatal bacterial meningitis may be underestimated as it is very hard to determine accurately due to a range wide of factors, which include: the difficulty in diagnosing neonatal meningitis, variations between hospital-based and community studies, regional differences and unregistered deaths in areas where the access to proper health care is poor or almost inexistent (Osrin et al., 2004). Moreover, although most data evidence derives from developed countries, the major burden of neonatal sepsis and meningitis occurs in the developing world (Furyk et al., 2011), where health care and data acquisition are unevaluated. Thus, it is obvious that the existing data does not reflect the true incidence of bacterial meningitis.

In industrialized developed countries, bacterial meningitis incidence is approximately 0.3 per 1,000 live births (Brouwer et al., 2010). The World Health Organization (WHO) estimates that there are 5 million neonatal deaths per year and that approximately 98%, an overwhelming majority, occurs in developing countries (Furyk et al., 2011). Neonatal meningitis contributes significantly to this shocking statistics, as it is recognized as one of the top ten causes of infection-related death worldwide (Fauci, 2001), with 126,000 cases annually and more than 50,000 deaths (Stoll, 1997, Weber et al., 2003).

Mortality from neonatal meningitis in developing countries is estimated to be 40-58%, against 10% in developed countries (Furyk et al., 2011). A study conducted throughout Asia reported an estimated incidence of neonatal meningitis from 0.48 per 1000 live births in Hong Kong, whereas in Kuwait, an incidence rate of 2.4 per 1,000 births (Tiskumara et al., 2009). In another study focused in neonatal infections in Africa and in South Asia, an incidence of neonatal meningitis ranging from 0.8 to 6.1 per 1,000 live births was reported (Thaver and Zaidi, 2009).

Group B *Streptococcus* is, beyond the neonatal period, the most common cause of bacterial meningitis, accounting for approximately 80% of cases among those less than 2 month of age (Schrag, 2011). *Escherichia coli i*s the second pathogen (Klinger et al., 2000) and *Listeria monocytogenes,* the third, and it possesses the unique characteristic of transplacental transmission (Heath et al., 2003). In Portugal, GBS is the most common isolate in early onset neonatal sepsis. A survey from 2008 in Portuguese infants younger than 90 days, estimated an overall incidence of invasive GBS disease of 0.54 per 1,000 live births, with a mortality rate of 6.6% (Neto, 2008). Moreover, this incidence also varies from one geographic area to another, as approximately 35% of pregnant women from the north are colonized with GBS, contrasting with the 13% found in the south (Neto, 2008).

Table 1 summarizes the most common pathogens responsible for central nervous system (CNS) infections and meningitis.

Table 1 – Pathogens commonly causing central nervous system infections in humans

Pathogen	Reference
***Streptococcus agalactiae* (Group B *Streptococcus*)**	(Brochet et al., 2008, Heath et al., 2009, Edmond et al., 2012)
Escherichia coli	(Klinger et al., 2000, Gaschignard et al., 2012)
Listeria monocytogenes	(Heath et al., 2003, Fayol et al., 2009)
Streptococcus pneumoniae	(O'Brien et al., 2009)
Neisseria meningitidis	(Kurlenda et al., 2010)
Haemophilus influenzaetype b	(Watt et al., 2009)
Mycobacterium tuberculosis	(Farinha et al., 2000)
Borrelia burgorferi	(Avery et al., 2005)
Candida albicans	(Aleixo et al., 2000)
Trypanossoma spp.	(Finsterer and Auer, 2013)

Although the mortality rate in developed countries declined almost from 50% in the 1970's to approximately 10% in the late 90's (Berardi et al., 2010), the morbidity rates are still high, as meningitis remains a major source of disability and long term sequelae (Puopolo et al., 2005). Neonatal meningitis survivors are at risk for developing moderate to severe disability, which include: problems in language, motor function, hearing, vision and cognition and also 5 to 20% have future epilepsy. Besides, survivors may also present more subtle symptoms: visual deficits, middle-ear disease and behavioral problems (Bedford et al., 2001). In a 2001 study, regarding a sample superior to 1500 neonates from the England and Wales with surviving until the age of 5, several neuro-motor disabilities were reported as cerebral palsy (8.1%), learning disability (7.5%), seizures (7.3%) and hearing problems (25.8%) (Bedford et al., 2001).

1.3 Streptococcus agalactiae

Streptococcus agalactiae is a Gram positive encapsulated bacterium which appears as diplococci or as a chain (Doran and Nizet, 2004, Nandyal, 2008). It is a catalase negative, beta-hemolytic microorganism and it is a facultative anaerobic,

growing preferentially in oxygen absence. During the 30's, Rebecca Lancefield observed that hemolytic *Streptococcus* from human and animal origins could be divided and distinguished serologically according to their polysaccharide capsule composition (Lancefield, 1933, 1934a). Moreover, five *Streptococcus* groups were then discovered: A, B, C, D and E (Lancefield, 1934b). In line with this type of classification, the most common designation for *S. agalactiae,* Group B *Streptococcus* (GBS), was implemented. Lancefield was also able to sub-divide GBS into different serotypes using the antigen immunogenicity of the polysaccharide capsule as criteria (Lancefield, 1934b). Currently there are ten known GBS serotypes: Ia, Ib, II, III, IV, V, VI, VII, VIII (Doran and Nizet, 2004, Shet and Ferrieri, 2004, Slotved et al., 2007).

Still during the 30's, Lancefield and Hare isolated GBS for the first time from the vaginal tract of a woman after having birth [reviewed by (Mulder and Zanen, 1984)] and three years later, Fry observed cultured GBS collected from women during delivery. Despite these facts, little knowledge of GBS role in neonatal infections was obtained during the next thirty years. It was only in 1964 that Eichkoff highlighted the importance of this pathogen in the previously stated subject. In the late 70's, GBS outstripped *E. coli* as the most important cause of newborn septicemia in the United States of America. Nowadays, GBS continues to be one of the major pathogens responsible for neonatal bacterial meningitis and septicemia (Stoll et al., 2011).

The Lancefield division does not demonstrate the true biodiversity regarding the existing GBS strains, as strains that belonging to different serotypes can be more related at a genetic level than strains from the same serotype (Davies et al., 2004, Tettelin et al., 2005).

A more profound knowledge about serotype relations was needed and multilocus sequence typing (MLST) was applied. This is a very important molecular biology technique and it is used to type multiple loci. This procedure characterizes microbial isolates using the DNA sequences of internal fragments of housekeeping genes (Davies et al., 2004). Approximately 450-500 bp internal fragments of each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. For each housekeeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type (ST). After the characterization of the GBS MLST profile, it was observed that isolates with the same sequence can actually belong to different capsular serotypes. From all known serotypes, serotype III seems to be the most virulent and it is very closely associated with most of the cases of neonatal meningitis (Nandyal, 2008).

2. GBS Disease

2.1 Risk Factors

GBS maternal colonization is the primary risk factor for the development of bacterial infections (Verani et al., 2010). The gastrointestinal tract is a natural reservoir for GBS and is likely the source of vaginal colonization (Dillon et al., 1982, Hoogkamp-Korstanje et al., 1982). In addition, a study suggested that the vagina becomes colonized with GBS as a result of its transfer from the rectum into the vagina, stating that rectal GBS colonization is a major predictor and risk factor for vaginal colonization (Meyn et al., 2009). Up to 30% of both sex individuals are commonly colonized with this bacteria in the gastrointestinal and genital tracts but remain asymptomatic. Importantly, 30% of the asymptomatic individuals are pregnant women colonized in the vaginal tract (Verani et al., 2010). Moreover, the carriage rate in the vaginal and rectal microbiota ultimately ranges between 10 to 37% and it's the same in both developing and developed countries (Bergeron et al., 2000, Verani and Schrag, 2010).

GBS colonization during pregnancy can be transient, intermittent, or persistent (Lewin and Amstey, 1981, Hoogkamp-Korstanje et al., 1982, Hansen et al., 2004). Some women with GBS colonization during a pregnancy will be colonized during subsequent pregnancies (Cheng et al., 2008, Turrentine and Ramirez, 2008). Nevertheless, previous delivery of an infant with invasive GBS disease is a risk factor for early-onset disease in subsequent deliveries (Carstensen et al., 1988, Faxelius et al., 1988, Schrag et al., 2002b). In addition to maternal colonization with GBS, other parameters increase the risk for neonatal infections such as: gestational age inferior to 37 completed weeks, abnormally longer duration of membrane rupture, intra-amniotic infection, young maternal age, black race, and low maternal levels of GBS-specific anti-capsular antibody (Baker et al., 1981, Boyer et al., 1983, Schuchat et al., 1994, Schuchat et al., 2000, Zaleznik et al., 2000, Oddie and Embleton, 2002, Adair et al., 2003).

2.2 Early-Onset and Late-Onset Disease

Epidemiologically, neonatal GBS disease is divided in two distinct forms, early-onset disease (EOD), occurring between 0-6 days after birth, and late onset disease (Barichello et al.), starting after the first week of life until day 89 (Phares et al., 2008).

Infants with GBS EOD generally present respiratory distress, apnea, or other signs of sepsis within the first 24–48 hours of life (Franciosi et al., 1973, Baker, 1978). The most common clinical syndromes of early-onset disease are sepsis and pneumonia but even though less frequently, early-onset infections can lead to meningitis. The case-fatality ratio of EOD has declined from as high as 50% in the 1970s (Baker and Barrett, 1974). In contrast, the progression of the infection to a state of meningitis, which is the most common manifestation of LOD, did not decline (Nandyal, 2008). Early-onset infections develop mainly *in utero* because GBS is able to ascend to the amniotic cavity, contaminating the amniotic fluid (Desa and Trevenen, 1984). Also, neonatal infection can be acquired during labor through exposure to GBS present in the vagina of a colonized woman (Katz and Bowes, 1988).

In relation to GBS LOD cases, the route of infection is not well known. It can be acquired from birth, as approximately 50% of the infants with LOD are colonized at delivery time with the same GBS serotype as the mother (Dillon et al., 1987), or it can be acquired from environmental sources (Berardi et al., 2013b). Horizontal transmission during the perinatal period may occur from mother to infant or from hospital or community sources (Melin, 2011). Another reported source of infection is breastfeeding (Godambe et al., 2005, Gagneur et al., 2009), although this has been under discussion in the scientific community as opinions differ (Berardi et al., 2013b). Nevertheless, it is thought that the bacterium is acquired by swallowing of infected maternal vaginal secretions during birth, leading to a persistent infection that culminates in its dissemination and in the later development of meningitis (Nandyal, 2008).

As illustrated in Figure 1, the first focus of infection is the lungs, as both vaginal fluid and amniotic fluid can be inspired. GBS caused pneumonia is characterized by severe pulmonary lesions, with bacterial infiltrates, hemorrhage and protein exudates into the alveolar space (Doran and Nizet, 2004). Thereby, lungs are an important gateway for bacterial infections, giving access to the bloodstream. The main consequences are bacterial dissemination to other anatomical places, such as the liver; bacteremia and septicemia and the consequent penetration into the brain (Doran and Nizet, 2004, Maisey et al., 2008).

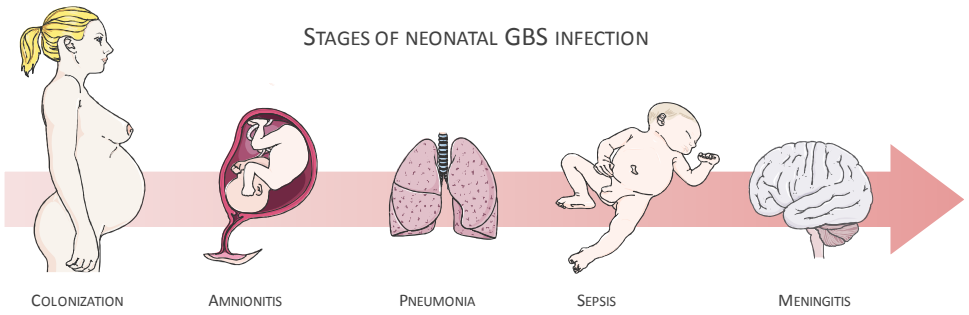

Figure 1 | Schematic view of the stages of neonatal GBS infection

Approximately 75% of the EOD cases are associated with type Ia, III and V serotypes (Nandyal, 2008). Serotype III GBS is the main responsible for the majority of infections in neonates worldwide (Edmond et al., 2012).

In LOD, it has been shown that most cases are associated with a singular capsulated serotype III GBS clone, designated as ST-17 (Jones et al., 2003, Lin et al., 2006, Tazi et al., 2010). The ST-17 clones are referred has being from the hyper-virulent type (Davies et al., 2004). Around 25-50% of GBS meningitis surviving infants experience permanent neurologic sequelae, including mental retardation, cerebral palsy, seizure activity, deafness and/or blindness (Edwards et al., 1985, Gibbs et al., 2004). Yet, these findings are most likely to underestimate the real incidence of GBS-induced meningitis, as signs of meningitis are often subtle in the neonate and therefore difficult to diagnose (Garges et al., 2006).

2.3 Prophylaxis for neonatal GBS Infections

In 1996, the Centers for Disease Control and Prevention (CDC), in association with both the American Congress of Obstetricians and Gynecologist and the American Academy of Pediatrics, presented guidelines for *intrapartum* antibiotic prophylaxis (IAP). According to these new measures, all pregnant women between the 35th and 37th week must be subjected to GBS screening and, whether positive, they are administered with IAP. In addition, the guidelines were meant for all the women that presented, at time of birth, risk factors of disease transmission (Verani et al., 2010).

In 2002, these guidelines were reviewed (Schrag et al., 2002a) and, in November of 2010, a new CDC document was emitted (Verani et al., 2010). Some key-points in the combat against GBS disease were reinforced, such as: upgrading and improving the laboratorial methods for rapid and more accurate GBS identification, change in the penicillin dose used in prophylaxis, alternate antibiotic options for penicillin allergic women, actualized IAP and screening algorithms for preterm delivery

women, and lastly algorithm improvement for newborn babies with risk of contracting EOD (Verani et al., 2010, Schrag and Verani, 2013).

Such measures were accepted worldwide in developed countries and, although they aided the reduction of EOD rates, they did not contributed significantly for the lowering of the LOD rates (Verani et al., 2010, Berardi et al., 2013a, Schrag and Verani, 2013). Actually, in the period of widespread IAP use, the incidence of invasive early-onset GBS disease in Active Bacterial Core surveillance (ABCs) decreased by more than 80% from 1.8 cases/1000 live births in the early 1990s to 0.26 cases/1000 live births in 2010 (Figure2) (Schrag and Verani, 2013). However, GBS meningitis among patients under 2 months of age did not change in a significant fashion after the introduction of universal GBS screening of pregnant women and improvement in neonatal intensive care (Thigpen et al., 2011). Furthermore, the casualties from both EOD and LOD remain high and the morbidity associated with LOD has not changed substantially over decades (Gibbs et al., 2004) (Figure 2).

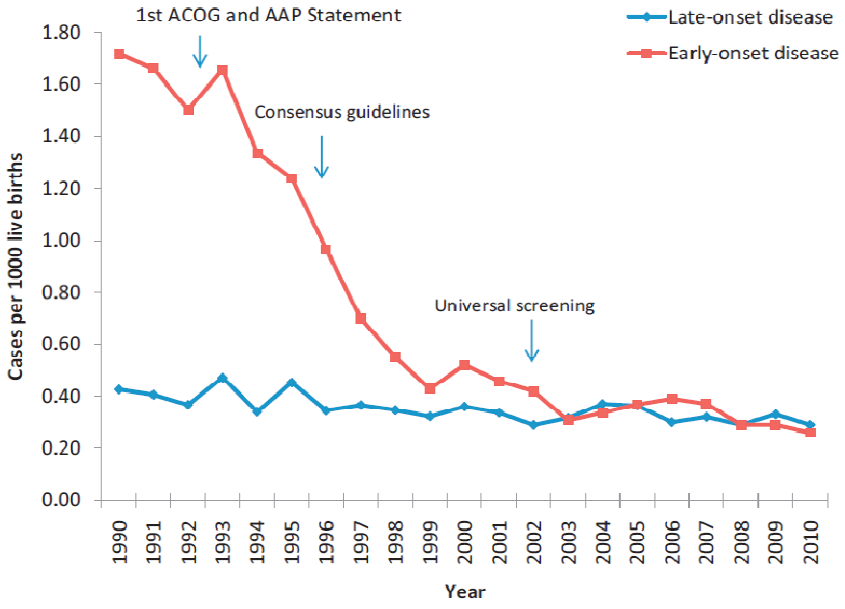

Figure 2 | Incidence of invasive early and late-onset group B streptococcal disease, Active Bacterial Core surveillance, United States, 1990–2010 (Schrag and Verani, 2013).

3 Meningitis

3.1 Pathophysiology of Meningitis

Bacterial meningitis is an inflammation of the meninges that affects the pia, the arachnoid and the subarachnoid space (Hoffman and Weber, 2009). It is a complex mechanism that occurs in a series of steps which include: the adherence of the pathogen to the host mucosal surfaces and subsequent colonization; the bacterial invasion to adjacent intravascular spaces; and, of course, the survival and multiplication inside the host, resulting in bacteremia. Once microorganisms reach the bloodstream, they can cross into the subarachnoid space and they can enter in places where the Blood Brain Barriers (BBB) is vulnerable or inexistent, such as the choroid plexus (CP) (Daum et al., 1978). In addition, bacteria leaving cerebral capillaries enter the cerebrospinal fluid (CSF) and initiate an inflammatory cascade in the subarachnoid space (Spellerberg, 2000).

Although bacteremia is very important for meningitis development, it's not sufficient by itself to microorganism penetration into the Central Nervous System (CNS). To cause meningitis, the pathogen must be able to leave the bloodstream and cross the barrier that separates it from the CNS.

3.2 Homeostatic Barriers of the Brain

The CNS is protected at three key sites: the endothelium of brain parenchymal blood capillaries, the choroid plexus epithelium and the arachnoid epithelium of the meninges (Abbott, 2005). These barriers play critical roles in controlling the movement of a series of metabolites, but also drugs, between the blood and the brain (Saunders et al., 2013).

Figure 3 displays the locations of the different homeostatic barriers in the brain, with (A) representing the Blood-Cerebrospinal Fluid Barrier (BCSFB); (B) the Blood-Brain Barrier (BBB); (C) the fetal CSF-Brain Barrier and (D) the Outer-CSF Barrier.

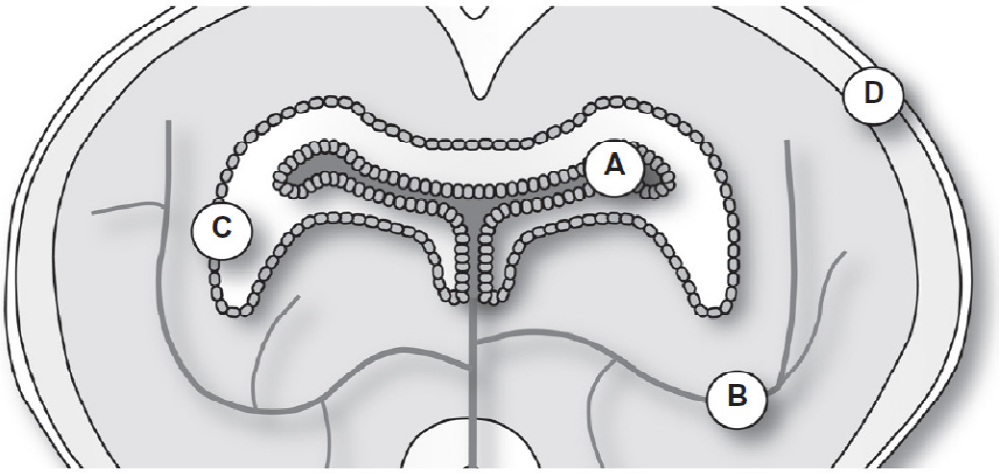

Figure 3 | Homestoatic Barriers of the Brain. (A) The blood-CSF barrier between the choroid plexus blood vessels and the CSF. (B) The Blood-Brain Barrier between the lumen of the cerebral endothelium and the brain parenchyma. (C) The inner CSF-brain barrier, present in only during development, between the CSF and neuroependyma. (D) The outer CSF-brain barrier between the subarachnoid space and overlying structures. [Adapted from (Saunders et al., 2013)].

The BBB is a functional and structural barrier which separates the brain extracellular fluid (BECF) from the blood in the CNS. As shown in Figure 4, three cellular elements of the brain microvasculature compose the BBB: brain microvascular endothelial cells, astrocyte end-feet, and pericytes (Kim, 2003, Ballabh et al., 2004).

The BBB establishes both a physical as well as a metabolic barrier, isolating the CNS from systemic circulation and thus creating a unique and stable environment for an optimal neuronal activity (Correale and Villa, 2009). Moreover, this structure maintains the neuronal microenvironment homeostasis in a biochemical fashion, as endothelial cells regulate the molecular traffic in the brain, protecting it from microorganisms and circulating toxins in the bloodstream (Kim, 2008).

Endothelial cells present unique characteristics that make them so efficient in their functions of protection. Tight junctions, found between the adjacent cerebral endothelial cells, form a diffusion barrier, which selectively excludes most blood-borne substances from entering the brain (Ballabh et al., 2004). Furthermore, their cytoplasm lacks fenestrations present in most of the peripheral tissues (Correale and Villa, 2009). These special features make the BBB virtually impermeable to ions and molecules. In addition, the almost inexistent pinocitosis also contributes to the tight control of the molecular trafficking (Correale and Villa, 2009).

While endothelial cells are the main keepers of the brain homeostasis, astrocytes and pericytes help maintaining the barrier properties, but their functions are not fully understood. Yet, evidence from cell culture studies indicate that astrocytes upregulate many BBB features: they tightly sustain the vessel wall and appear to be critical for the induction and maintenance of the tight junction barrier (Hayashi et al.,

1997, Sobue et al., 1999). As for pericytes, they are not believed to function as a barrier in the mammalian brain (Ballabh et al., 2004).

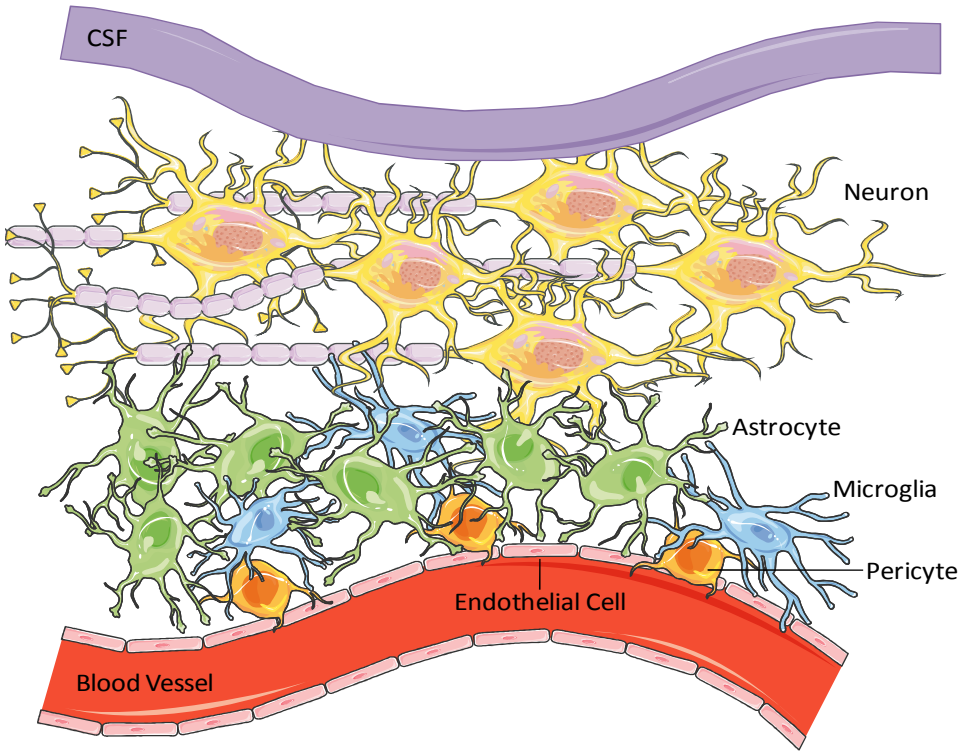

Figure 4 | **The blood–brain barrier**. The blood–brain barrier is formed by brain microvascular endothelial cells, astrocytes and pericytes. It maintains the neural microenvironment by regulating the passage of molecules into and out of the brain, and protects the brain from any microorganisms and toxins that are circulating in the blood.

The BCSFB displays fundamentally different properties when compared with the BBB, either structurally or functionally (Johanson et al., 2011). The CP operates jointly with blood brain barrier, as the choroid epithelial interface on the BCSFB has distinctive structural features that facilitate regulatory functions for ultimately promoting neuronal environment maintenance. The endothelium in the choroid plexus is fenestrated and forms a non-restrictive barrier. In addition, choroid plexus epithelial cells are joined by functional tight junctions towards the apical surface that stop the movement of hydrophilic molecules (Saunders et al., 2013). Two prominent areas where the blood is near the CSF are the arachnoid membrane blanket over the subarachnoid space and the CP invagination of the ventricles. The lack of true lymphatic capillaries in the inner surface of the brain makes the CSF the main cleanser of harmful proteins and anionic catabolites in young adulthood (Johanson et al., 2011). Interestingly, inner CSF-brain barrier (Figure 4) is present only during early development, between the CSF and the neuroependyma (Dziegielewska et al., 2000). In adulthood due to a loss of strap junctions and due to the switch from neuropendyma

to ependyma, this structure is not present. So, there's no restriction of molecular trafficking in the adult brain across this interface.

Apart from the described barriers, another barrier plays an important role in the regulation of neuronal homeostasis, the outer CSF-brain barrier (Figure 4). This barrier is located between the CSF-filled subarachnoid space and overlying structures (Saunders et al., 2013). Endothelium is fenestrated and therefore its functions as barrier are not the most efficient. On the other hand, outer cells of the arachnoid membrane are connected by tight junctions.

3.3 Pathogen Translocation across the Blood Brain Barrier

Theoretically, all pathogenic microorganisms are able to access the CNS but only a small fraction of these pathogens cause infections in the CNS (Kim, 2008). The BBB relies on a very complex equilibrium that maintains the neuronal environment homeostasis. The microorganism crossing and invasion reflect a series of tight interactions between the host and pathogen. Simultaneously, bacteremia and consequent bacterial translocation to the BBB (Spach and Jackson, 1999) are also a route of CNS infection. Even though high levels of bacteria are important for meningitis development, it is not enough on its own for microorganism entrance in the CNS (Kim, 2003).

Microorganisms can bypass the BBB by three different methods [reviewed by (Kim, 2006) and (Drevets et al., 2004)]: transcellularly, paracellularly and/or by means of infected phagocytes, so called Trojan horse mechanism (Figure 5). Transcellular traversal, refers to bacterial entrance thought the cells without tight junction rupture of the BBB; it has been demonstrated for several bacterial pathogens, such as *E. coli* (Kim, 2001, 2002, Kim et al., 2003, Kim, 2003), GBS (Nizet et al., 1997), *Streptococcus pneumoniae* (Ring et al., 1998), *L. monocytogene*s (Greiffenberg et al., 1998), *Neisseria meningitidis* (Unkmeir et al., 2002), and for some fungal pathogens such as *Candida albicans* (Jong et al., 2001). As for the paracelular mechanism, it has been defined as microorganism passage between the endothelial cells of the BBB, with or without tight junction destruction. Paracellular penetration of the BBB has been suggested for the protozoans *Trypanosoma spp*. (Grab et al., 2005). Lastly, in the Trojan horse mechanism, infected phagocytes carry the pathogen through the BBB and into the CNS; this mechanism has been suggested for *L. monocytogenes* (Join-Lambert et al., 2005) and *Mycobacterium tuberculosis* (Drevets et al., 2004). Recently our group showed that recruitment of the host plasminogen to the GBS surface

generates a proteolytic bacterium that after conversion to plasmin, can traverse the BBB (Magalhaes et al., 2013).

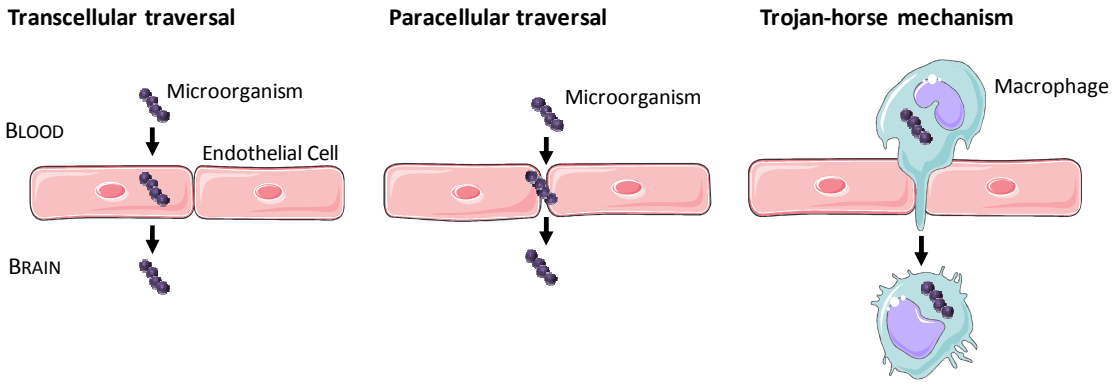

Figure 5 | **Mechanisms involved in microbial traversal of the blood–brain barrier.** Pathogens can cross the blood–brain barrier transcellularly (Transcelullar traversal), paracellularly (Paracelullar traversal) and/or in infected phagocytes (the Trojan-horse mechanism).

3.4 GBS Infection and Virulence

The CNS tropism that some GBS strains present and its ability to invade the CNS reflects a series of complex interactions between the host cells and many surface-associated and secreted bacterial components. Briefly, some GBS molecules, like fibrinogen-binding protein A (FbsA) (Tenenbaum et al., 2005), pili A (PilA), (PilB) (Maisey et al., 2007), laminin-binding protein (Lmb) (Tenenbaum et al., 2007), β-hemolysin/cytolysin toxin (β-h/c) (Doran et al., 2003), serine-richrepeat-1 (van Sorge et al., 2009), and glycosyltransferase involved in the production of a cell-membrane glycolipid anchor for lipoteichoic acid (Doran et al., 2005), mediate interaction of the pathogen with brain microvascular endothelial cells (BMEC) and penetration through the BBB. Many of these GBS ligands are known to bind to extracellular matrix molecules such as fibronectin, fibrinogen and laminin, which successively bind host-cell-surface proteins such as integrins. It has been recently identified a hypervirulent GBS adhesin (HvgA), which significantly impacts on intestinal colonization by translocation across the intestinal barrier and mediates adhesion to BMEC (Tazi et al., 2010).

3.5 GBS translocation across the BBB

Once in the bloodstream, GBS has the ability to cross the BBB and cause meningitis. The mechanisms from which GBS establishes CNS infection remain unknown. Some studies state that GBS is able to penetrate the BBB through a transcelullar mechanism, also described to *E. coli* (Kim et al., 2003), since GBS was observed intracellularly within vaculae connected to human BMEC membranes (Nizet et al., 1997). Electron microscopy also shows that GBS migrates through the cell, from the apical surface to the basal surface with no sign of free bacteria in the cytoplasm. GBS requires the presence of live cells to cross the BBB, as protein and nucleic acid synthesis is necessary and also modifications of the cytoskeleton, microtubules and microfilaments of the host cell are needed (Huang et al., 2000). However, conducted studies in other culture-types, such as respiratory and gastrointestinal epithelium, indicate that the bacteria could enter through a paracelullar mechanism, once GBS is found between adjacent cells (Pezzicoli et al., 2008). We recently showed that hijacking of the host plasminogen system to generate a proteolytic bacterium constitutes another mechanism used by GBS to penetrate into the brain (Magalhaes et al., 2013)

3.6 Experimental Models of Neonatal Meningitis

Concerning bacterial meningitis, namely the induced by GBS, there's a lack of clinical data, as sampling procedure is often invasive and complex. The limitation of a suitable animal model, essential for a better understanding of the pathogenesis and pathophysiology of neonatal meningitis induced by this microorganism could explain the worldwide high mortality and morbidity rates caused by GBS infection (Gaschignard et al., 2011). Most of the data is derived from severe cases of neonatal meningitis, normally associated with death or severe health complications of the studied individuals (Brochet et al., 2008). In addition, the current knowledge about the pathogenic mechanisms that contribute to CNS complications and neuronal damage are largely derived from experimental models of meningitis, either *in vitro* or *in vivo*. However, none of the experimental animal models developed to date mimic the route of infection used by GBS in humans. Although these studies are valuable, as they helped to understand some points of this microorganism pathogenesis, they have serious limitations and drawbacks, as they bypass the normal bacteremia-meningitis sequence of GBS infection (Ferrieri et al., 1980, Mancuso et al., 1994, Leib et al., 1996, Reiss et al., 2011, Patterson et al., 2012, Barichello et al., 2013).

Therefore, the aim of my project was the characterization of our animal model of neonatal GBS infection that uses an approach that addresses human pathogenesis of ascending infection of neonates acquired from the lower genital tract of their mothers.

Objectives

Objectives

The main aim was to characterize the suitability of our animal model to the study of neonatal meningitis induced by GBS. Thus, in this project the specific aims were:

- To assess vaginal and gastrointestinal tracts maternal colonization and GBS infection on their offspring;
- To evaluate the meningitis characteristics signs in the brains of the pups born from colonized mothers;
- To evaluate the cognitive and motor performance of the animals that survive to GBS infection, in adulthood.

Materials and Methods

Bacterial strains and growth conditions

GBS strain BM110, capsular serotype III, and MLST sequence type ST-17 is a well-characterized isolates from human with invasive infections. GBS BM110 was cultured at 37℃ in Todd-Hewitt (TH) broth or agar (Difco Laboratories) containing 5 µg/mL of colistin sulphate and 0.5 µg/mL of oxalinic acid (*Streptococcus* Selective Supplement, Oxoid).

Animals and Ethics statement

Six- to eight-week-old male and female BALB/c mice were purchased from The Jackson Laboratory. All animals were kept at the ICBAS animal facilities during the time of the experiments. All procedures were performed according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 123) and 86/609/EEC Directive and Portuguese rules (DL 129/92). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Gestation time and pregnancy monitoring

Detection of the vaginal plug and measurement of body weight were jointly used to determine the time of gestation. Two to three females were put together with one male and examined for the presence of vaginal plug every morning. The finding day of the vaginal plug was considered as gestation (G) day one (G1) and the pregnancy progression was monitored every other day by weighting the females.

Neonatal mouse model of GBS-induced meningitis

Pregnant BALB/c mice were intra-vaginally (i.vag.) infected at G17 and G18 with 10^6 GBS BM110 cells, in a 30 µL volume. This infection period was determined to be the optimal, as early infections did not allow pregnancy to reach term (data not shown). Pregnant females were allowed to deliver and newborns were kept with their mothers during the entire time of the experiment. Survival curves were determined in a 30-day period. Pups were weaned after postnatal day (PND) 30. To assess bacterial colonization, the liver, lungs and brain were aseptically removed at the indicated PND and homogenized in PBS. Serial dilutions were prepared in sterile saline, plated on TH agar and incubated overnight at 37℃.Blood was collected in heparinized containers

and centrifuged to collect the sera. When possible, 10 µL of blood were saved for CFU counts. The sera were stored at -80˚C until analysis. At the indicated times, stool samples were collected and serial dilutions were plated on selective medium (Granada, Biomérieux) for CFU counts.

DNA extraction

Stool Samples

Stool samples were placed in 200 µL pre-heated PBS and subsequently homogenized. Samples were centrifuged, 2 min, at 8000 rpm (Biofuge Fresco, Heraeus Instruments) and the supernatant was rejected. The pellet was resuspended in 150µL in TE (Tris 10 mM, EDTA 1 mM) solution and centrifuged, 10 min, at 8000 rpm. The supernatant was discarded and the pellet resuspended in 150 µL of TE-Sucrose (Tris 10 mM, EDTA 1 mM, 7% Sucrose) solution. 60 µL of Lysozime (Sigma) (10 ng/mL) were added and incubated for 1h, in water bath, at 56ºC, with periodical shaking. 36 µL of EDTA (Merck) 0.25 M+ 24 µL SDS 10% (Sigma) solution was added and the solution was resuspended several times. 40 µL of Proteinase K (Affimetrix) (5 mg/mL) were added, gently mixed and incubated during 30 min at 37ºC (until solution appeared clear). 27µL of NaCl (Merck) 5 M was added and gently mixed. 37.5 µL of CTAB (previously heated) was added and incubated for 20 min at 65ºC. Genomic DNA was obtained through Phenol-Chlorophorm (Sigma) extraction. Samples were centrifuged and the aqueous phase was transferred to a new tube and precipitated in isopropanol (Merck) and shaken until a DNA precipitated was formed. Thereafter, 0.5 mL of ethanol (Sigma) was added, samples were centrifuged, the supernatant was discarded and DNA was rehydrated in 50 µL of DNA-Hydration Solution (Qiagen).

PCR assays

The *cfb* gene encoding the Christie-Atkins-Munch-Petersen (CAMP) factor is virtually present in all GBS isolates, and therefore is suitable for PCR detections (Wilkinson, 1977). The GBS *cfb* gene (CAMP factor) was amplified from the extracted genomic DNA. Specific primers were used in the PCR assay: *Sag59* (TTTCACCAGCTG TATTAGAAGTA) e Sag190 (GTTCCCTGAACATTATCTTTGAT) (Ke et al., 2000). *Escherichia coli* K1 was used as a negative control. In addition, purified GBS genomic DNA was used as a positive control. MyTaq HS Red Mix (Bioline) and ultrapure H_20

(Gibco) were used in the PCR mix. MWG Biotech thermocycler was used according with Table 2. 1.5% ultra-pure agarose (Life Sciences) gel electrophoresis was performed to analyze amplification products.

Table 2 |GBS-specific conventional PCR assay characteristics.

	Cycles	Temperature(˚C)	Time
Denaturation	1	94	3 min
		95	1 seg
Amplification	40	55	30 seg
		72	2 min
Storage	--	8	∞

Histopathology

Brain sections were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. 4-5 µm-thick sections were cut and stained with hematoxylin and eosin (H&E).

Behavioral assessments

The mice that survived to GBS-induced meningitis were evaluated to determine their cognitive and motor performance. The behavioral tests were performed in a sound attenuated, temperature (21 ± 1ºC) and humidity (70%) controlled room with a 12-h light–dark cycle (lights at 7 a.m.), using male animals at PND60 (begin of adulthood), from different litters. The animals were kept at approximately 90% (26.64 ± 1.28 g) of their free feeding body weight and began training after reaching this weight. During the whole test time mice had restricted access to food (they were only fed following testing) and their weight and general health were carefully monitored every day to prevent more than a 10% body weight loss.

Radial maze test

The cognitive status of the animals as regards spatial learning and memory was assessed with the 8-arm radial maze. Working and reference memory were assessed simultaneously through a fixed position reward task, in which half of the arms were baited and their positions were fixed throughout the training trails. The radial maze consists in a central area (22 cm in diameter) giving access to eight equally-sized arms in transparent acrylic (length, 25 cm; width, 6.5 cm). Identical food wells (2.5 cm deep

and 3 cm in diameter) were placed at the distal end of each arm. Commercialized sugar pellets (Bioserv F0042 - DPP'S 45MG SUGAR 50TH) located at the end of each baited arm were used as rewards. Two days prior to the beginning of the test, the rewards were placed once a day in the animal cages, to allow them to explore and eat the pellets. There were extra-maze clues including free-standing laboratory equipment, and geometric pictures in walls to help mice navigation. A digital stopwatch was used to record the amount of time taken to a mouse to complete a trial.

Habituation/training - On the first habituation day, each animal was placed alone in the center of the starting platform and allowed to freely explore the maze. On the second habituation day, the mice were allowed to explore the apparatus with randomly placed food pellets throughout the maze, for a 5 min period. On the third habituation day, the food pellets were placed at the distal end of each arm and the animals were allowed to explore for 5 min. The habituation period ended after the mouse ate at least four rewards or when 5 min had elapsed.

Test – During the test phase, four out of eight arms were baited and randomly assigned for each mouse, but always constant for the same animal throughout the trial period, and the mice were given a maximum of 5 min to complete the maze. Geometrical figures placed on the walls and the researcher were the only visual extra maze cues present. In each training session, the mouse was placed within a transparent cylinder on the platform in the middle of the maze for few seconds. The cylinder was then lifted and the animal was allowed to move freely in the maze. It was considered that an animal had entered an arm when the four paws and the tail were inside it. Trials ended when the animal had either eaten all four rewards or 5 min had passed, whichever came first. Once the animal returned to the central platform and the cylinder was lowered; a minute later the next trial took place. One session of two trials was performed per day over fourteen days. The maze was rotated and wiped with a dextran solution at 2% between animals to eliminate or reduce olfactory cues from different individuals.

Behavioral analysis - There were four measures used in behavioural analysis. The number of errors made: working memory error, defined as re-entering an arm already visited within a trial; and a reference memory error, defined as entering a never baited arm. Trial completion time was also included. It started when the cylinder was lifted, in which the mouse had full access to explore the maze, and stopped when the animal entered the fourth baited arm. Response latency was also measured and defined by the total session duration divided by the number of arms entered (seconds per entry). Seven blocks were calculated by average measure of four trials per day.

Open field (OF)

Exploratory behavior and general locomotor function were assessed in the open field. In each session, the mice were placed in the center of the open-field arena (40 x 40 x 40 cm). The OF arena is divided virtually into 16 equal squares, via a 4 x 4 grid to assist with data analysis. Each session on the OF was registered for 5 minutes. All trials were recorded by a video camera (SONY DCR-SF 290), suspended above the test arena, and analyzed afterwards using the software package Observer XT 7.0 (Noldus Information Technology). The following parameters were assessed: total distance walked, frequency and time spent in center or periphery section of the OF. The number of rears (i.e. rat reared on its hind paws, both on or off the walls), arena exploration and time spend unmoved were also scored. The apparatus was cleaned between subjects with a solution of 2% dextran.

Neurotransmitter Determination

After the completion of behavioral studies, mice were euthanized by decapitation and the brains rapidly dissected on ice, frozen on dry ice and stored at -80°C until neurochemical analysis. Amino acid levels and the levels of monoamines and their metabolites were measured by high performance liquid chromatography, combined with electrochemical detection (HPLC/EC), using a Gilson instrument (Gilson, Inc., Middleton, WI, USA) (Alves et al., 2009).

Amino acids

For gamma-aminobutyric acid (GABA) and glutamate, the prefrontal cortex, hippocampus, striatum and cerebellum were used. The day before the neurochemical determination, tissues were thawed, and homogenized in 200 µL of ice-cold 150 mM potassium buffer with phosphoric acid, through ultrasonication (Sonifier W-250, Branson Ultrasonics) and centrifuged at 16,000 g, for 10 min at 4ºC. The supernatant was collected, diluted 1:2 in dilution solution (0.4 N perchloric acid, 0.4 mM sodium dissulfite, 0.9 mM EDTA), filtered thought a 0.2 µm nylon microfilter (Corning) at 16,100 g for 5 min, at 4°C, and stored at -20°C overnight. Afterwards, samples were derivatized by adding 100 µl of NaOH 0.1N and 15 µl OPA (10 mg/mL of OPA, 45.4 M sodium sulphite, 4.5% absolute ethanol in 327 mM borate buffer at pH 10.4) to 50 µL of sample. Samples were allowed to react at room temperature in the dark, for 10 min and then injected into the HPLC system. The mobile phase consisted of 0.06 M sodium dihydrogen phosphate, 0.06 mM EDTA and 20% methanol, pH adjusted to 4.4 with

Phosphoric acid and it was filtered and degassed. The flow was maintained at 0.8 mL/min. Concentrations of GABA and glutamate were calculated using a standard curve generated with a glutamate and GABA standard (Sigma-Aldrich). Final results were expressed in terms of amino acid content per amount of protein.

Monoamines and their metabolites

Levels of norepinephrine (NE), epinephrine (E), dopamine (DA), 3,4-diydroxyphenylacetic acid (DOPAC), 5-hydroxyindoleacetic acid (5-HIAA), serotonin (5-HT) and homovanillic acid (HVA) were determined in the striatum, prefrontal cortex, hippocampus and amygdala. The different brain regions were ultrasonicated in 200 µL of ice-cold 0.2 M perchloric acid and centrifuged at 13,000 rpm for 3 min at 4 ℃. The supernatant was then collected and filtered as described above. Aliquots of 120 µL were injected into the HPLC system, using a mobile phase of 70 mM of potassium phosphate monobasic buffer (pH adjusted to 3.0 by adding phosphoric acid) in 10% (v/v) of methanol, 1 mM 1-heptanosulfonic acid and 107.5 mM Na-EDTA. The flow was maintained at 0.8 mL/min. Concentrations of neurotransmitters were calculated using standard curves generated with standard monoamines (Sigma). Final results were expressed in terms of monoamine content per amount of protein.

Total protein determination

Protein content was determined using the BCA Protein Assay Kit (Thermo Scientific-Pierce). Briefly, bovine serum albumin (BSA) was used as a standard protein (0.01– 0.5 mg/mL). 10 µL of sample or BSA standard in duplicate were loaded into a microplate and added 200 µL of working reagent to each well. After 30 min incubation at 37ºC, absorbance was read at 562 nm using a microplate reader.

Statistical analysis

All graphs were generated using GraphPad Prism software (GraphPad Software). Means and standard errors of the means (SEM) were calculated. Survival studies were analyzed with the log-rank test and bacterial counts were analyzed using Mann-Whitney *U* test. The data from radial maze test were submitted to analyses of variance (ANOVA) with repeated measures for block sessions. Each block-session was calculated by average measure on four successive trials. For statistical analysis of

open-field data a one-way ANOVA was used, followed by Tukey post-hoc comparisons, when appropriate. Student's unpaired *t* test was used to analyze the differences between groups, when appropriate. A *P* value of < 0.05 was considered statistically significant.

Results

1 Vaginal and gastrointestinal tracts of the female mice remains colonized with GBS after delivery

It is known that GBS transmission to newborns usually occurs during the labor by aspiration of contaminated fluids present in the vaginal mucosa. Taking into account, pregnant BALB/c female mice were infected intra-vaginally with $5x10^5$ CFU of GBS BM110, a serotype III GBS hypervirulent strain ST-17, responsible for the overwhelming majority of neonatal meningitis (Jones et al., 2003).

The 17th and 18th gestational days were defined as the specific window of intervention for intra-vaginal inoculation of the bacterium and the vaginal colonization was assessed by plating the vaginal washout several times after the delivery. Because the excess of blood and bodily fluids at delivery day made the procedure very difficult, this day was excluded from evaluation. As shown in Figure 6A, all the females remained highly colonized until day four after birth. After this day, the bacterial loads started to decrease and by day 10, no GBS was detected. Since the gastrointestinal tract is a reservoir of GBS, the bacterium was also searched in the feces of these female mice. PCR assays with specific GBS-primers (Ke et al., 2000) were used to search for GBS in the feces. As shown in Figure 6B, GBS was found in their feces, 30 days after birth indicating that the bacterium colonized the gastrointestinal of these females.

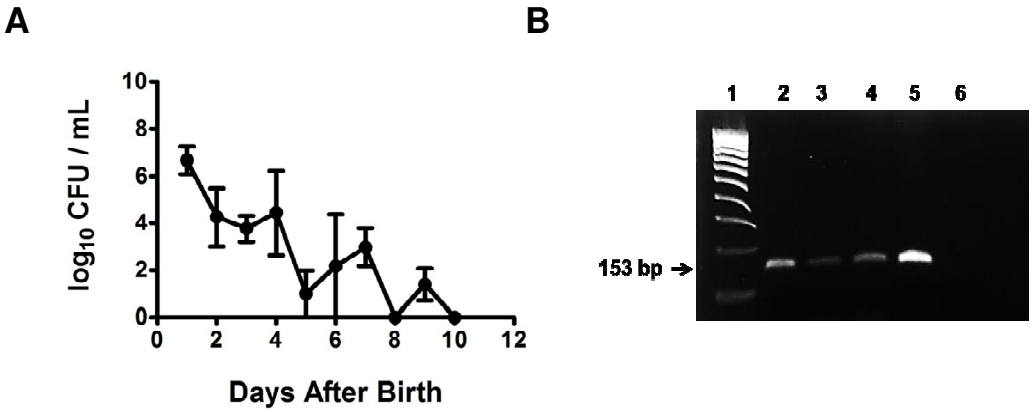

Figure 6 | **Colonization of the murine vaginal mucosa with GBS.** Pregnant BALB/c mice were intra-vaginally infected with $5x10^5$ CFU of GBS BM110, at the 17th and 18th gestational day. (A) Upon birth, the number of GBS colony-forming units (CFU) in vaginal tract was determined, by vaginal washouts with PBS. Data are the mean (n=20) of 10 independent experiments ± SEM. (B) Conventional PCR assay for the detection of GBS (153-bp amplicon) in anal specimens from BALB/c female mice. Lane 1 shows a 100 bp ladder molecular-size standard. Lanes 2, 3 and 4 samples collected 30 days after delivery. Lane 5 and 6 shows positive and negative controls, respectively.

2 GBS is vertically transmitted to the progeny

The survival rates of mice born from infected females were evaluated and as shown in Figure 7, approximately 40% of the offspring died after birth with 14% dying within the first 24h after birth. After day four, no more deaths were registered.

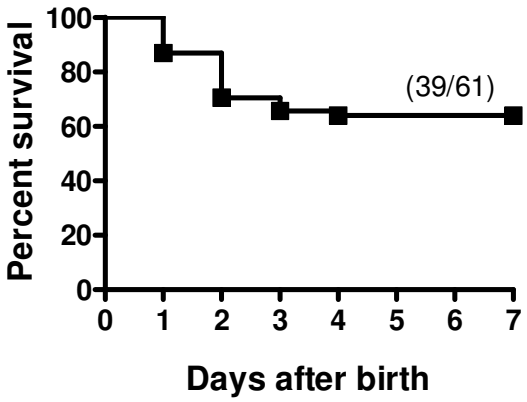

Figure 7 | Murine model of neonatal GBS-induced meningitis. Pregnant BALB/c female mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17^{th} and 18^{th} gestational days. Kaplan-Meier survival curves of neonatal mice born from intra-vaginally infected dams, monitored during a 7 days period. The numbers between parentheses represent the number of animals that survive versus the total number of infected progeny. Results represent data pooled from ten independent experiments.

The bacterial load was assessed in key organs such as the lungs, the liver, blood and brain at the indicated post natal days (PND). As shown in Figure 8A, newborn mice presented high and sustained level of GBS in the lungs, up to one week after birth. Moreover, GBS was also detected in the blood of infected pups reaching a maximum level at PND1, decreasing daily afterwards and by PND7 bacteria were no longer detected (Figure 8B). In addition to the presence of GBS in the bloodstream of infected pups, the liver was also used to search for GBS and the results showed that bacterial load was detected from PND1, decreasing until it and at PND14 was no longer detectable (Figure 8C). Interestingly, GBS was also found in the brain of these animals (Figure 8D) reaching the highest colonization at PND4. This last data showed that GBS traversed the BBB and reached the central nervous system, causing infection.

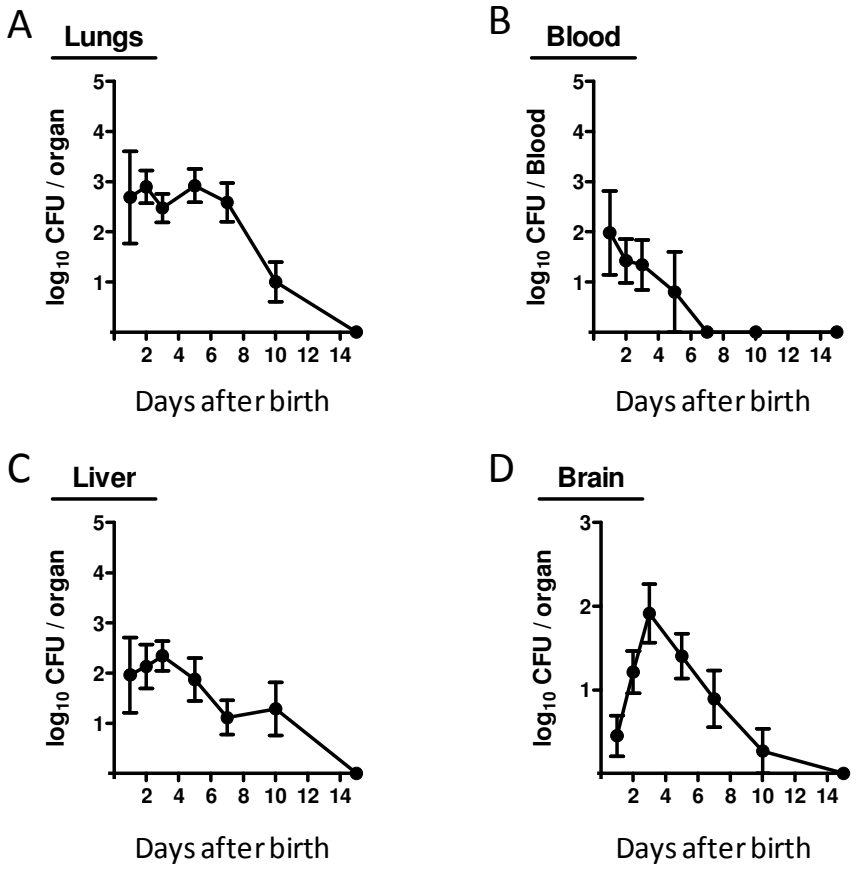

Figure 8 | Murine model of neonatal GBS-induced meningitis. Bacterial counts of GBS in lungs (A), blood (B), liver (C), and brain (D) of neonatal mice at different time points after birth. Data points are shown as the mean (n=16) pooled from 3 independent experiments ± SEM.

3 Brain histopathological changes in GBS infected neonates

To further analyze the brain of infected pups, histopathological analysis was performed. All infected pups exhibited some clear hallmarks of meningitis: meningeal detachment, edema, venous congestion, and signs of hemorrhage (Figure 9). These histological features are in accordance with those observed in humans. Moreover, influx of inflammatory cells was also observed in the brains of GBS-infected pups, when compared to age-matched control animals. These results are indicative that our murine model of neonatal meningitis mimics the vertical transmission of infection similarly to the observed in humans.

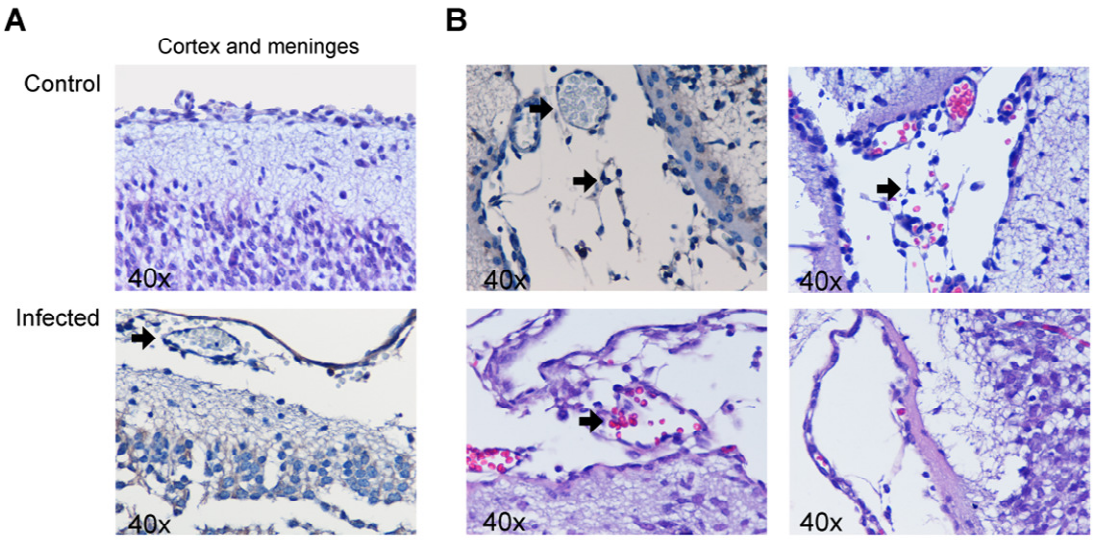

Figure 9 | Murine model of neonatal GBS-induced meningitis. Histopathology of brain tissues of representative individual pups infected with GBS compared with non-infected control, after staining with H&E. Arrows indicate meningeal detachment, edema, and venous congestion. Magnification, 40x.

4 Mice survive to neonatal GBS infection present behavior alterations in adult life

Cognitive and motor performance of the offspring that survived to GBS infection was performed in adult life to assess whether they presented neurological sequelae, as it has been described in humans.

4.1 Memory and learning performance

The outcome of GBS infection on learning and memory performances were verified by testing their ability to learn a given task in the radial arm maze apparatus. Differences among the two studied groups, age-matched non-infected controls and GBS-survivors, were observed when comparing the first session with the last one (Figures 10A-B). In the control group, a significantly decreased average errors over the days were observed, indicating learning. In contrast, the number of working and reference memory errors in the GBS-survivors was not significantly different over the days, meaning that they were unable to learn the task. Moreover, there were significant differences among the groups concerning to latency to enter in the first arm in the first four block sessions (Figure C). GBS-survivors showed meaningful slower arm choices responses than non-infected controls.

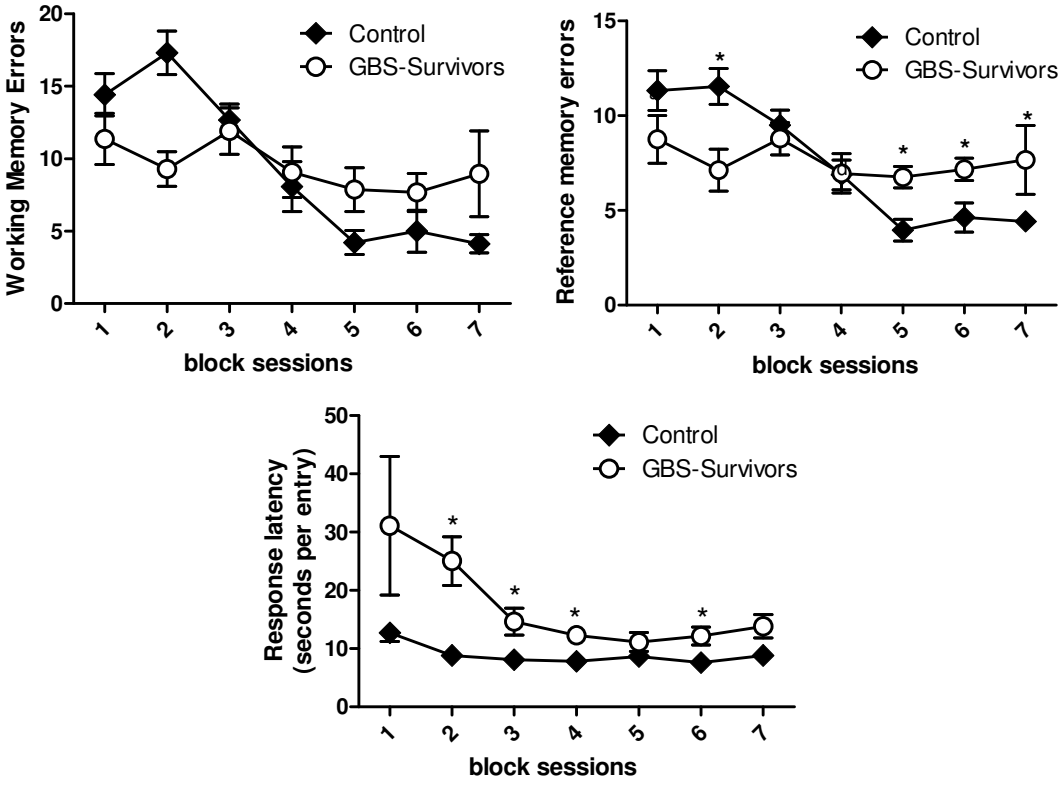

Figure 10 | Effects of neonatal GBS-induced meningitis on learning and memory performances in adulthood. Pregnant BALB/c female mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17th and 18th gestational days. Offspring that survived to infection, or non-infected controls, were examined by their ability to execute tasks in the radial arm maze at PND60. Number of reference (A) and working (B) memory errors, and latency (C) in the radial arm maze task. The data represent the mean ± SEM. *, $P < 0.05$.

4.2 Motor performance

To assess whether the increase latency was associated with reduced mobility, the mice were subjected to the open field test (OFT). The OFT is widely used to evaluate the mice's natural exploratory behavior and general motor function, as it opposes the mouse's innate curiosity to explore a unknown area with the fear of wide-open spaces (Stanford, 2007). Figure 11 illustrates the total distance covered by the two mouse groups, GBS-survivors travelled significantly less when compared with age-matched controls. Moreover, Figure 11 shows the overall activity of GBS-survivors, revealing that these animals spent significantly more time in the periphery compared with non-infected controls. Also, these animals present a decreased central ambulatory activity comparatively with age-matched controls. The frequency in movement, either into the periphery or towards the center area was significantly decreased in the GBS-survivors group (Figure 12A and 12B). Also, when analyzing the exploratory activity of GBS-survivors, the results showed that this group of animals had a meaningful higher

immobility and frequency when compared with age-matched controls (Figure 12D). In addition, exploratory vertical rearing of the mice was also assessed, and Figure 12C shows that the GBS-survivor group spent significantly less time rearing compared with non-infected animals.

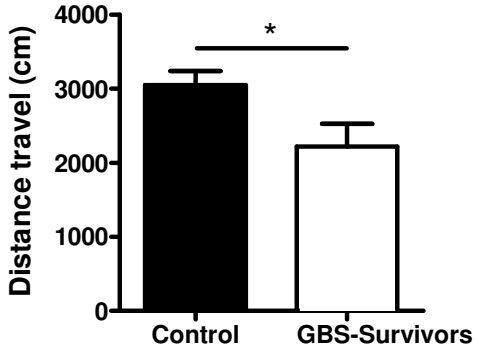

Figure 11 - Effects of neonatal GBS-induced meningitis in the locomotor and exploratory behaviors in adulthood, distance travelled in OFT. Pregnant BALB/c female mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17th and 18th gestational days. Offspring that survived to neonatal GBS infection, or non-infected controls, were tested in an open field (OF) apparatus at PND60.

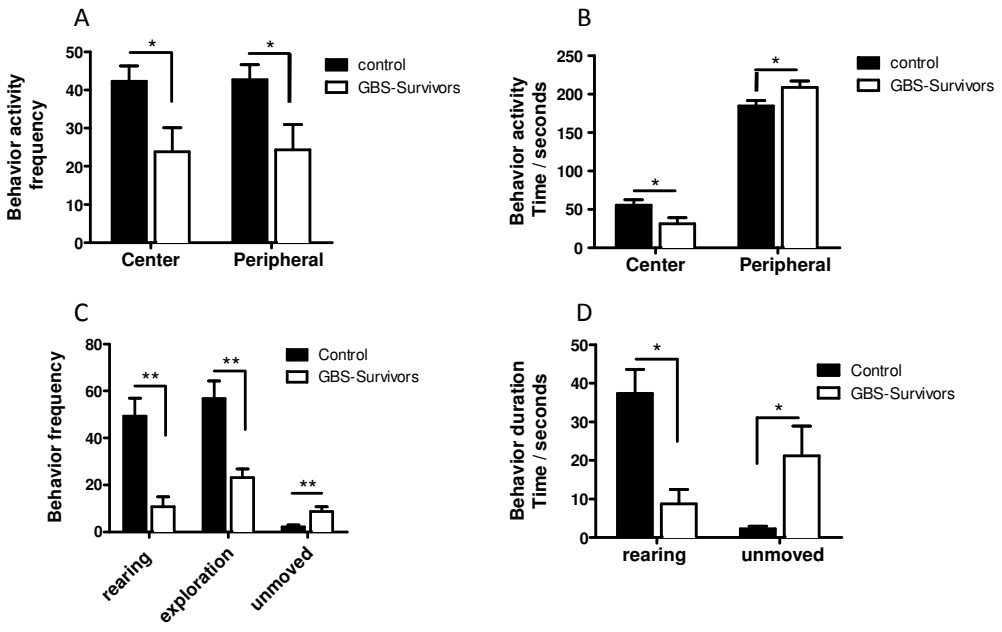

Figure 12 | Effects of neonatal GBS-induced meningitis in the locomotor and exploratory behaviors in adulthood. Pregnant BALB/c female mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17th and 18th gestational days. Offspring that survived to neonatal GBS infection, or non-infected controls, were tested in an open field (OF) apparatus at PND60. (A-B), frequency and time spent in the periphery and center of OF maze; (C-D) frequency and time of exploratory behavior. Data are the mean ± SEM, *P<0.05; **P<0.001.

Rearing and exploratory frequency were analyzed as an index of investigative behavior, and GBS-survivors present a significantly decrease in rearing and exploration activities comparatively to non-infected controls.

All these results showed that the locomotor and exploratory abilities of GBS-survivors were decreased.

5 GBS-induced meningitis leads to an altered neurotransmitter pattern

To further confirm the differences in the behavioral profile of the GBS-survivors group, the levels of the neurotransmitters (amino acids and monoamines) were quantified and interpreted to see whether they could explain such behaviors. Thus, several brain areas were submitted to a HPLC-ED analysis, namely brain sections related with memory acquisition, learning and motor activity.

5.1 Amino acid quantification

For amino acid quantification both glutamate and *gamma*-aminobutyric acid (GABA) were analyzed in the hippocampus, thalamus, cerebellum and pre-frontal cortex. Concerning the glutamate concentration in the hippocampus, a significant decrease in its levels was observed in the GBS-survivor group when comparing with the non-infected controls (Figure 13), which supports the behavioral tests in the radial maze arm.

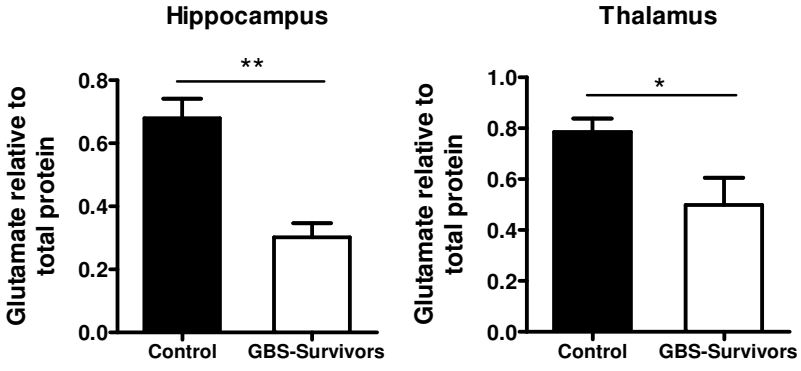

Figure 13 | Effects of neonatal GBS-induced meningitis in glutamatergic function. Pregnant BALB/c mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17^{th} and 18^{th} gestational days. Levels of Glutamate in the hippocampus and in the thalamus of the offspring that survived to neonatal GBS infection, or non-infected controls, were determined by HPLC-EC, at PND60. Relative levels are shown and normalized to total proteins. Data are the mean + SEM, from 8 mice per group. *P<0.05; **P<0.001

In addition, the levels of glutamate in the thalamus are also decreased in the GBS-survivor animals (Figure 13), which could probably explain the motor deficits observed in the OFT. Glutamate levels in the cerebellum and in the pre-frontal cortex did not present differences (data not shown). The levels of GABA in the different brain sections did not show significant differences (data not shown).

5.2 Monoamine Quantification

Several monoamine neurotransmitters: epinephrine, norepinephrine, dopamine (DA), serotonin and both their metabolites were quantified in hippocampus, striatum, pre-frontal cortex and amygdala. When observing the hippocampus of GBS-survivors, the levels of the neurotransmitter DA were significantly decreased compared with non-infected controls (Figure 14A). Accordingly, the levels of the DA metabolite, 3,4-dihydroxyphenilacetic acid (DOPAC), were also significantly decreased in the hippocampus of GBS-survivors (Figure 14B). Homovallinic acid (HVA) levels although analyzed, they were below the detection limit (data not shown).

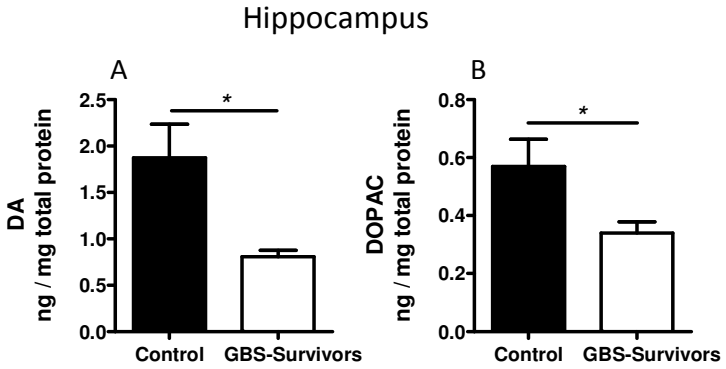

Figure 14 | Effects of neonatal GBS-induced meningitis in dopaminergic function in the hippocampus. Pregnant BALB/c female mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17^{th} and 18^{th} gestational days. Levels of DA (A), and DOPAC (B) in the hippocampus of the offspring that survived to neonatal GBS infection, or non-infected controls, were determined by HPLC-EC, at PND60. HVA levels were below the detection limit (data not shown). Relative levels are shown and normalized to total proteins (A-B). Data are the mean + SEM, relative to total proteins. *P<0.05.

The levels of DA neurotransmitter in striatum were slightly lower in the GBS-survivor than in controls animals, with no significant differences (Figure 15A). DOPAC, A significant decrease of DOPAC in the striatum of GBS-survivors were observed (Figure 15B). The same tendency is observed with HVA, another DA metabolite, as it is significantly decreased in the GBS-survivor animals Figure 15C.

The levels of DA and its metabolites were also analyzed in the pre-frontal cortex and in the amygdala, but no significant differences were obtained (data not shown). Moreover, the levels of epinephrine, norepinephrine, serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) were analyzed in all brain sections, but no significant differences were observed (data not shown).

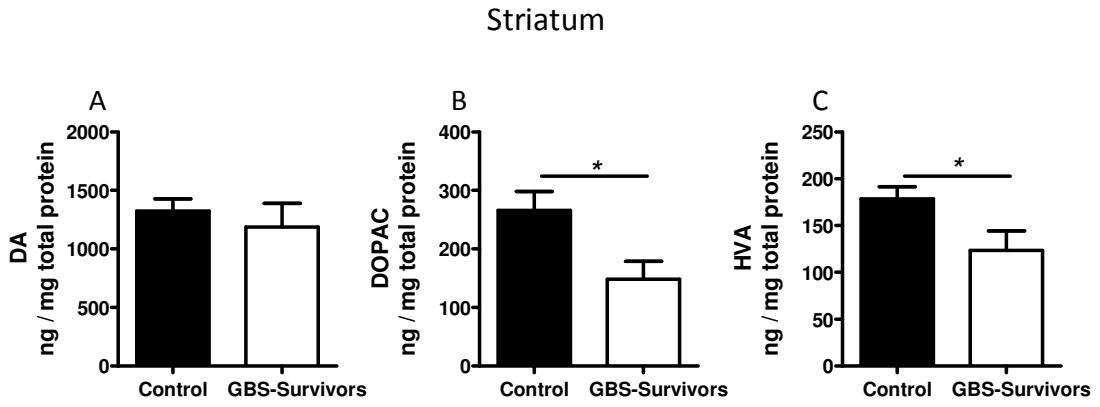

Figure 15 | **Effects of neonatal GBS-induced meningitis in dopaminergic function in the striatum**. Pregnant BALB/c mice were intra-vaginally infected with 10^6 CFU of the ST-17 hyper virulent strain BM110, in the 17^{th} and 18^{th} gestational days. Levels of DA (A), DA metabolites DOPAC (B) and HVA (C) in the striatum of the offspring that survived to neonatal GBS infection, or non-infected controls, were determined by HPLC-EC, at PND60. Relative levels are shown and normalized to total proteins (A-C). Data are the mean + SEM, from 8 mice per group. *P<0.05.

Discussion

Discussion

Central nervous system infections, namely meningitis, continue to be an important cause of morbidity and mortality throughout the world. Indeed, bacterial meningitis is still one of the top ten causes of infection-related deaths worldwide (Scheld et al., 2002). Group B *Streptococcus* is the leading causative agent of bacterial meningitis, accounting for 86.1% of cases among those less than 2 month of age (Schrag, 2011). The incomplete understanding of GBS-induced meningitis pathogenesis is the one important contributing factor for the high rates of mortality and morbidity associated with this disease worldwide.

Clinical data concerning neonatal meningitis cases are scarce and arduous to obtain, as newborn babies are very difficult to diagnose correctly. In addition, the sampling methods related with meningitis diagnosis are, in most cases, invasive and uneasily procedure, causing pain and discomfort to patients. Apart from all, the few available meningitis data concerning humans is derived from serious cases of infections and worse yet, it is often obtained from fatal cases (Brochet et al., 2008). Therefore, it is easy to understand that most of the meningitis related data is attained from cell culture assays, *in vitro* and from experimental animal models, *in vivo*.

When searching in the literature, we can find a wide range of experimental GBS-induced meningitis models, using different animals as models, such as rats (Reiss et al., 2011), mice (Mancuso et al., 1994) or more recently the zebra-fish (Patterson et al., 2012). However, all these models use routes that do not address the human pathogenesis of ascending infection to neonates, as they bypass the normal bacteremia-meningitis sequence (Ferrieri et al., 1980, Mancuso et al., 1994, Leib et al., 1996, Barichello et al., 2013). One particular example is the study from Reiss and his colleagues at 2011, where GBS meningitis was induced through intracisternal injection, directly into the brain of 7 and 11-day old rats (Reiss et al., 2011). Not only the infection route is artificial, but also the age of the animal is not the most appropriated, as 7 and 11-day old for rats are no longer considered in the neonatal period. Regardless the stated drawbacks, these models contributed in a very important matter for the scientific advances in this area.

Therefore, the development of an experimental model of GBS-induced neonatal meningitis that mimics the human routes of GBS infection is of great importance. Such model will help to understand in a more profound way, the mechanisms whereby GBS can cause disease. This kind of insight will be very valuable for the development of scientific knowledge and also for the discovery of new therapeutic approaches.

In our experimental model, pregnant dams were infected intra-vaginally with GBS. It is known that GBS-mediated infection in human neonates usually occurs during passage through the vagina at birth. Figure 6A shows that female mice infected with GBS intra-vaginally remained highly colonized until day 4 after delivery. This showed that GBS is present in the vaginal tract of pregnant dams whilst the moment of birth ensuring that all neonatal mice contacted with the bacterium through infected mucosa.

The data regarding the survival rates of the infected offspring in our neonatal model showed that approximately 40% of the progeny died from GBS infection (Figure 7). Interestingly, the percentage of death observed in these pups is similar to what had been observed in human babies, prior to the implementation of IAP preventive measures (Phares et al., 2008).

GBS was found in the gastrointestinal tract of healthy individuals, as female mothers were positive for GBS in the gastrointestinal tracts by PCR analysis of their feces 30 days after birth (Figure 6B). This data sustains the idea that the gastrointestinal tract acts as a reservoir for GBS and so its prevalence in the rectum higher than in the vaginal tract (Schuchat, 1998). It has been described that the gastrointestinal tract acts a reservoir for GBS, and therefore it is the most likely source of vaginal colonization in humans (Dillon et al., 1982, Hoogkamp-Korstanje et al., 1982). Bacteria colonizing the gastrointestinal tract can migrate to other mucosal sites, in particular to the vaginal tract of pregnant women (Meyn et al., 2009). Based upon these new developments, universal screening and GBS sampling was set off to a new method, as combined samples of rectal and vaginal swabs are nowadays used (Block et al., 2008). Actually, a study suggests that the intestinal gateway might be involved for the initiation of LOD (Tazi et al., 2010). This is supported by the data that 60% to 40% of human babies are asymptomatically colonized with GBS at birth, and remain positive for those bacteria in the rectal tract for several weeks (Weindling et al., 1981).

In human babies, the main gateway to GBS infection is thought to be the lungs, as contaminated vaginal fluids can be aspirated during birth (Doran and Nizet, 2004). Accordingly, in our model, newborn mice had sustained levels of GBS in the lungs (Figure 8A), until one week after birth. Moreover, GBS were also found in the blood of newborn mice (Figure 8B) which indicates that GBS is able to cross the alveolar-capillary barrier and consequently access directly into the bloodstream. To confirm the systemic infection, liver was also analyzed and, as shown in Figure 8C, the livers of the offspring of infected mothers are colonized with GBS.

To cause CNS infections, pathogens have a long way to travel, as they must not only cross lung epithelial barriers and reach the bloodstream, but also gain access to the brain and thus to the CNS (Kim, 2006). Our experimental model shows that

bacteria were found in the brain of the pups born from colonized mothers (Figure 8D). This result indicates that GBS was able to traverse blood-brain barrier and reach the central nervous system, has it observed in humans. Moreover, meningitis hallmark-like features were displayed in histopathological brain slides, as GBS-infected pups presented meningeal detachment, edema, venous congestion, and signs of hemorrhage (Figure 9B). All these histological features are consistent with those described in human patients. In addition, an influx of inflammatory cells was also observed in the brain of the pups born from colonized mothers when compared with pups born from non-colonized mothers (Figure 9A). Even though no evidence of bacteria inside the brain parenchyma, it is known that brain damage is not necessarily caused by the direct entrance of bacteria into the brain (Hoffman and Weber, 2009). Actually, meningeal inflammation per se can be sufficient to explain morphological differences as the ones observed in our experimental model.

All of the above results show that our murine model of GBS-induced meningitis mimics the course of human pathogenesis of vertical transmission from mother to her offspring.

Even though the IAP measures were important to decrease the numbers of EOD, the cases of LOD remain unaltered (Schrag and Verani, 2013). Actually, up to 50% of the GBS LOD-survivors later on their lives are likely to experience a series of long-term neurological sequelae, including learning and memory disabilities (Edwards et al., 1985). To confirm whether some of the observed neurological sequelae in humans were also observed with mice from our experimental model, behavioral studies were conducted with non-infected controls and GBS-survivor mice. Interestingly, marked differences between the two animal groups were observed. GBS-survivor group presented overall inferior performance results both in the radial-arm maze and in OFT. Indeed, in the radial maze test, non-infected controls display a decrease in the number of errors committed, whereas GBS-survivors exhibited learning difficulties, as their number of working memory errors does not decrease significantly during the whole session period (Figure 10A).

A similar trend is also observed when concerning the reference memory, as GBS-survivors commit more errors than the non-infected control group showing a clear difficulty on learning (Figure 10B). Moreover, when assessing the motor condition of these GBS-survivor animals through the OFT, overall exploratory and locomotor abilities were significantly decreased. Indeed, GBS-survivors spent more time without moving (Figures 12A-D) or when moved, they spent it on the periphery, a synonym for latency (Figure 12B) than the non-infected group. One could also interpret the previous results as anxiety signs, and even though OFT can be used in the context of anxiety

studies, further and more suitable tests are needed to confirm this hypothesis. Overall, our behavior results are in accordance with the described outcomes of GBS-induced meningitis in humans. A recent study showed that in spite of the reduced mortality, serious long term sequelae result from GBS-LOD. This report was in accordance with a paper from 1985, as data showed interesting similarities. Both these studies displayed data from GBS-induced meningitis survivors, all of them evaluated at the age of 6, and from this group, one half presented global or mild-to-moderate mental retardation, from which learning disabilities, and language deficits with associated neurological abnormalities. In a 2001 prospective study from England and Wales in 1996-97, neonates surviving until the age of 5 presented: cerebral palsy (8.1%), learning disability (7.5%), seizures (7.3%) and hearing problems (25.8%). In addition, the same study stated that severe disability was significantly more common in infants with GBS-induced meningitis when compared with other meningitis-inducing pathogens (Bedford et al., 2001).

Since GBS-survivors presented inferior performance in the behavioral tests, next we tried to understand whether differences in the neurotransmitter pattern could explain the behavioral profile observed. For that purpose, amino-acid and monoamines levels were analyzed in different brain sections associated both with learning and memory acquisition and motor control. Glutamate is the primary excitatory neurotransmitter in the mammalian CNS as it is crucial for learning and memory formation. Figure 14 shows that GBS-survivors present decreased levels of glutamate in the hippocampus, which could explain the difficulties observed in task learning (Figure 10). The hippocampus is involved in spatial processing, memory formation, cognitive function and mood regulation (Riedel and Micheau, 2001), thus its functional impairment may be reflected in a series of learning and memory disabilities found in children that survive meningitis. Learning, memory and neural plasticity depend on highly regulated patterns of neuronal activity, which are tightly controlled in time and space. When a disturbance occurs, it triggers an unbridled, unregulated and excessive neuronal activation, resulting in function impairment. The immune system, when working properly, that is, when tightly controlled, plays a crucial role in learning, memory, neural plasticity and neurogenesis, as cytokines have a modulatory effect on neuronal tissue [reviewed in (Yirmiya and Goshen, 2011)]. For instance, interactions between T cells and neurons in the hippocampus are important for memory consolidation; the high frequency stimulation-induced IL-1production plays a facilitator role in the development of the increased excitability that characterizes Long Term Potentiation (LTP), among others (Bliss and Collingridge, 1993). LTP is thought to be the main responsible cellular mechanism for learning and memory formation and it is a

long-lasting enhancement in signal transmission between two neurons, resulting from synchronous stimulation (Teyler and DiScenna, 1987). However, in a context of infection, when there's a higher production of pro-inflammatory cytokines, hyper-excitability of neuronal circuits can lead to seizures, excitotoxicity and neurodegeneration (Allan and Rothwell, 2001). For example in very young individuals, such as neonates, in which the brain is more plastic and the large number of newly generated neurons are more excitable. This makes newborn babies more susceptible to inflammation induced hyper-excitability and febrile seizures (Yirmiya and Goshen, 2011). In addition, it decreases the ability to induce LTP, among other mechanisms, and therefore it can result in memory and learning disabilities. Hyper-excitability, although not yet fully understood, could be a mechanism to help on explaining the shift between the beneficial function to a harmful one of the immune system. When in continuum, as it happens under inflammatory conditions, hyper-exicitability may widespread in the brain, and therefore be a brain damage causing factor (Ding et al., 2011). In addition, the dopaminergic system is usually associated with the reward system, but as well with movement control. Also, dopaminergic inputs into the hippocampus and frontal cortex are also relevant in learning, attention and decision making (Ernst and Paulus, 2005). Thus, when evaluating the levels of dopamine and its metabolites in the hippocampus of GBS-survivors, a significant decrease of both DA and DOPAC was observed, when comparing to age matched controls (Figure 14). This data set is also in agreement with the significant learning and memory disabilities found in children that survive to meningitis (Grimwood et al., 1995). Moreover, dopamine plays an important role in the nigrostriatal pathway, which connects the substantia nigra with the striatum (Graybiel et al., 1994, Schultz, 2002).It is particularly involved in the production of movement, as part of a system called the basal ganglia motor loop. Inputs from the cerebral cortex are received in the basal ganglia and substantia nigra (Schultz, 2002). The output of these two are the primary motor cortex and via the thalamus, the premotor cortex. The neurotransmitters GABA, glutamate and dopamine play central roles in the basal ganglia motor loop, as dopaminergic projects to the striatal region of the brain are also involved with motor control (Middleton and Strick, 2000). As shown in Figure 12, the mice that survived to neonatal GBS infection, presented reduced global activity, in accordance with the decreased levels of the DA metabolites, DOPAC and HVA, in the striatum (Figure 15), which indicate reduced DA metabolism. Of note, this can lead to a decreased influx of glutamate from the thalamus to the motor cortex and thus result in hypokinesia, which is characterized by an abnormal decrease in muscular movement (Cauli et al., 2009a). In accordance, we detected significantly lower glutamate levels in the thalamus of the GBS-survivor

animals (Figure 13). Interestingly, reduced glutamate levels were previously associated with hypokenesia in a rodent model of encephalopathy (Cauli et al., 2009b)

GBS-survivor mice presented a meaningful number of alterations in both learning and in spontaneous behavior. These data are in agreement with the observed decreased neurotransmitter levels in the brain sections of the same group of mice. Both glutamatergic and doparminergic function seem to be affect and thus influencing the normal function of the hippocampus and the strial-thalamic circuit as well.

Taking into account all the above results we can conclude that our mouse model mimics the human neonatal diseases induced by GBS infection.

Concluding Remarks

Concluding Remarks

The precise mechanisms by which GBS cause meningitis are not yet fully understood. Until now, all the existing models use infection routes that do not mimic the human pathogenesis of ascending infection of neonates as they bypass the normal bacteremia-meningitis sequence.

Our experimental model of GBS-induced meningitis is the first to mimic the natural course of human pathogenesis of neonatal infection through vertical transmission during gestation/birth. Indeed, we verified that pups born from GBS colonized mothers are infected during labor; these neonates present bacterial load in lungs, in blood, in liver and in brain. Moreover, the brains of infected pups showed the classical features of meningitis such as: meningeal thickening, cerebral bleeding and massive influx of inflammatory cells. In adulthood these mice present neurological sequelae, since they learn worse, are less active and curious than the non-infected controls. All these behavior alterations have been confirm by an altered neurotransmission pattern. Thus, all the obtained results were consistent with those observed in humans.

Moreover, this model brings new insights about LOD transmission, to our knowledge this is the first experimental study to report mother-to-child transmission. This will help explain LOD cases and the morbidity and neurological sequeale associated.

This novel experimental animal model could be used to explore and to characterize the pathophysiological mechanisms of the CNS inflammatory response and neuronal damage induced by GBS infection important for the development of new therapeutic approaches.

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